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Carbacaprazamycins: Chemically Stable Analogues of the Caprazamycin Nucleoside Antibiotics

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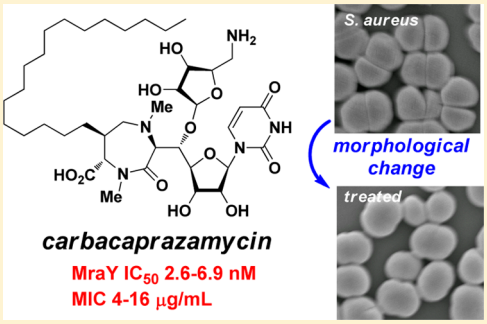
Supporting Information

ABSTRACT: Carbacaprazamycins, which are chemically stable analogues of caprazamycins, were designed and synthesized. These analogues were active against drug-resistant bacterial pathogens such as methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci, and their activities were comparable to those of the parent caprazamycins. The effect of treatment with carbacaprazamycin on morphological changes in *S. aureus* indicated that the mode of action was completely different from those of existing peptidoglycan inhibitors.



KEYWORDS: *antibiotics, drug resistance, MraY, caprazamycins, peptidoglycan*

The development of novel classes of antibacterials for systemic use, starting from sulfonamides in 1936, has been remarkably slow since the 1960s,[1] and only four classes of drugs with novel scaffolds have since been approved: linezolid in 2000,[2] daptomycin in 2001,[3,4] and fidaxomicin in 2011,[5] and bedaquiline in 2012.[6] The continued emergence of drug resistance to existing antibacterial agents represents a severe and ongoing public health concern, which demands the discovery of new antibiotics.[7] Eight of the 11 antibacterial drug classes introduced for clinical use are natural products or their derivatives, highlighting the important role that natural products have played in antibiotic discovery.[8,9] An important criterion in selecting among potentially promising natural product antibacterial agents is the synthetic tractability of the initial scaffold for the optimization of the potency and drug disposition properties to generate "lead" compounds.[10−14]

Caprazamycins (CPZs) (Figure 1, **1**), which are isolated from the culture broth of *Streptomyces* sp. MK730-62F2,[15,16] are members of a class of naturally occurring 6′-*N*-alkyl-5′-*β*-*O*-aminoribosylglycyluridine antibiotics including liposidomycins (LPSs, **2**), muraminomicin B (**3**), and A-97065.[17−23] CPZs have shown antimycobacterial activity in vitro not only against drug-susceptible (minimum inhibitory concentration, MIC = 3.13 μg/mL) but also against multi-drug-resistant *Mycobacterium tuberculosis* strains (MIC = 3.13 μg/mL), and they exhibit no significant toxicity in mice. CPZs are inhibitors of the phospho-MurNAc-pentapeptide translocase (MraY), which is responsible for the formation of lipid I during peptidoglycan biosynthesis.[24−26] Because MraY is an essential enzyme among bacteria, it is potentially a novel target for the development of antibacterial agents.[27,28] The unusual features of the molecules are a 5′-*O*-aminoribosyl-5′-*C*-glycyluridine moiety and a characteristic diazepanone that is attached to the lipophilic side chain. We previously succeeded in the total synthesis of caprazol, which is a core structure of CPZs and lacks the lipophilic side chain.[29,30] This synthesis strategy allowed us to study the structure−activity relationship (SAR) of CPZs with a range of analogues.[31−33] Although caprazol is completely inactive to bacteria, palmitoylcaprazol (**4**), which is a simplified analogue at the lipophilic side chain, exhibited excellent antibacterial activity against drug-resistant bacterial pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE).[34] Despite their promising biological activity, rather lengthy reaction steps were required to prepare **4**. In addition, the *β*-acyloxy ester found in the diazepanone moiety is prone to *β*-elimination during the synthesis, and we speculated that this could represent a metabolic liability.[35,36] Therefore, it is necessary to overcome this chemical stability. We designed analogues **5** and **6**, wherein the hydrophobic chain of the palmitoyl group was linked directly to the diazepanone via a C−C bond, as chemically stable analogues are devoid of the labile *β*-acyloxy ester moiety (Figure 2).[26] Herein, we describe the synthesis and antibacterial activity of **5** and **6**, termed carbacaprazamycins (cCPZs). Our previous SAR studies of CPZs revealed that the 3D orientation of the uridine, aminoribose, and lipophilic side-chain moieties is important to maintaining antibacterial activity, and diazepanone plays the role of a scaffold in attaching these key components.[29,30] Replacing the acyloxy substituent at the 3‴ position with the corresponding alkyl group could cause a conformational change in the diazepanone ring, changing the

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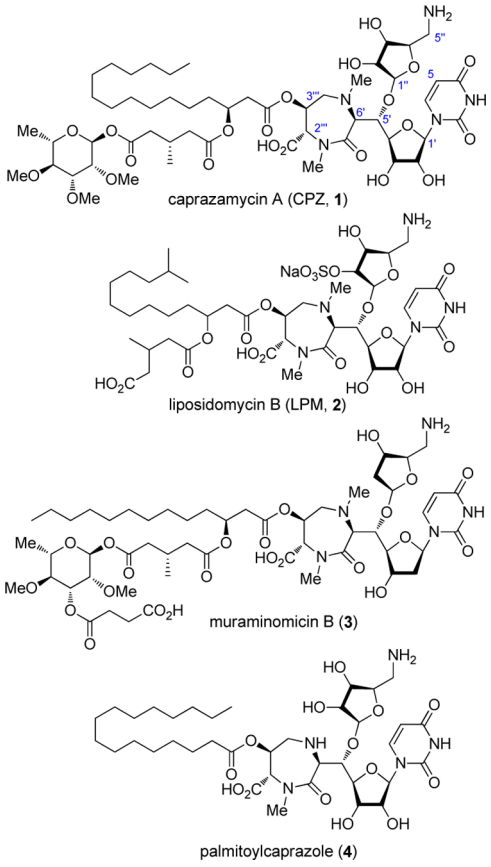


Figure 1. Structures of caprazamycins and their congeners.

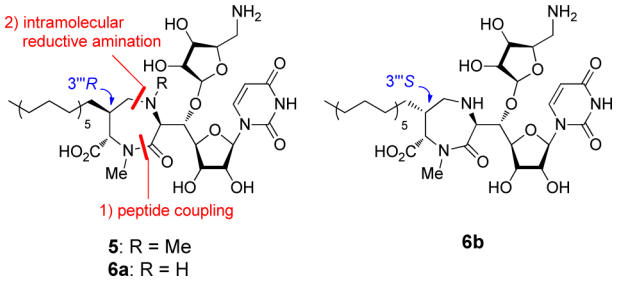


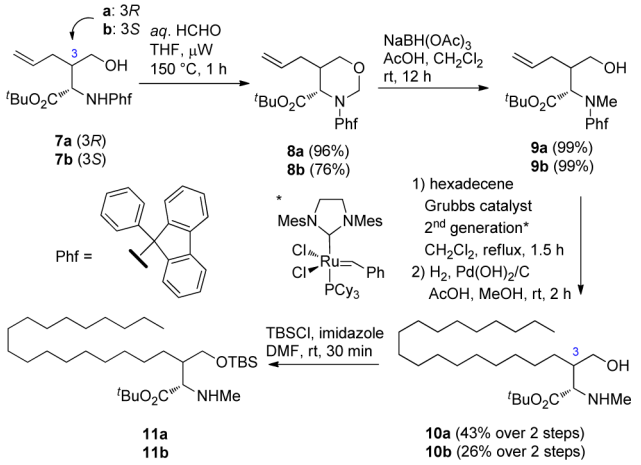
Figure 2. Structures and synthesis strategy of carbacaprazamycins.

3D orientation of the key substituents. Therefore, we sought to prepare **6b**, which is an epimer of **6a** at the 3‴ position. Our approach to the synthesis of cCPZs includes the late-stage construction of the diazepanone via (1) installation of a β-branched *N*-methyl-α-amino acid segment by peptide coupling and (2) intramolecular reductive amination to construct the diazepanone ring (Figure 2).

RESULTS AND DISCUSSION

First, (3*R*)-**10a** and (3*S*)-**10b**, which are key components for the construction of diazepanone, were prepared as shown in Scheme 1. (2*S*,3*R*)-*t*-Butyl 3-hydroxymethyl-2-phenylfluorenylamino-hex-5-enoate (**7a**) was prepared from L-aspartic acid γ-methyl ester in four steps according to a previously reported procedure.[37] Then, *N*-methylation was investigated after the formation of the oxazinane followed by reduction of the *N*,*O*-methylidene group.[38] Compound **7a** was first reacted with paraformaldehyde $(CHO)_n$ to form oxazinane **8a**; however, the reaction did not proceed. No improvement was observed even

Scheme 1. Preparation of 11ab



in the presence of an acid catalyst such as TsOH. The reduced reactivity of **7a** may be attributed to the steric hindrance of the phenylfluorenyl (Phf) group. Microwave (MW) irradiation promoted the reaction to afford the desired **8a** in 96% yield (150 °C, 1 h, 9 bar). The reduction of **8a** with $NaBH(OAc)_3$ in the presence of AcOH provided *N*-methylamine **9a** in quantitative yield. Treatment of **9a** with hexadecene in the presence of the Grubbs second-generation catalyst[39] afforded the cross-metathesis product, and the simultaneous reduction of the olefin and removal of the Phf group was achieved by catalytic hydrogenation to afford amine **10a** in 43% yield over two steps. Corresponding epimer **10b** was also prepared in a manner similar to the synthesis of **10a**. Note that **9b** was easily transformed to the corresponding γ-lactone via intramolecular transesterification. This also occurred during the transformation to **10b**. The resulting **10b** was also converted to the corresponding γ-lactone, which was the reason for the low chemical yield of **10b**. Therefore, the transformation of **9b** to **10b** should be conducted quickly, and the resulting **10b** should be used immediately after its preparation.

The synthesis of **5** and **6** through assembling **10** and **12**[29,30] followed by construction of the diazepanone is described in Scheme 2. Initial attempts to couple **10a** and **12** without the protection of the hydroxyl group of **10a** by 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide hydrochloride (EDCI) in the presence of various additives, such as 1-hydroxy-1*H*-benzotriazole (HOBt), 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3*H*)-one (DEPBT),[40] and *N*,*N*-dimethylaminopyridine (DMAP), failed to provide the desired secondary amide **15a**, and ester **13** was obtained instead. The *O* → *N*-acyl-migration of **13** was examined using a Lewis acid such as Et_2AlCl, Mg_2O, or $BF_3{\cdot}OEt_2$ in a variety of solvents and temperatures; however, none of these attempts were successful. Once the hydroxyl group of **10a** was protected with a TBS group (Scheme 1), coupling of the resulting **11a** with **12** by DEPBT and $NaHCO_3$ in THF proceeded smoothly to afford secondary amide **14a** in 51% yield. Upon removal of the TBS group, the liberated hydroxyl group of **15a** was oxidized by Dess–Martin periodinane[41] to furnish the aldehyde. The Cbz group was removed by catalytic hydrogenolysis to afford aminoaldehyde **16a**. Further treatment with $NaBH(OAc)_3$ in the presence of AcOH in CH_2Cl_2 yielded diazepanone **17a** in 73% yield over four steps. The secondary amine in the diazepanone ring was methylated by $(CHO)_n$ and NaBH-

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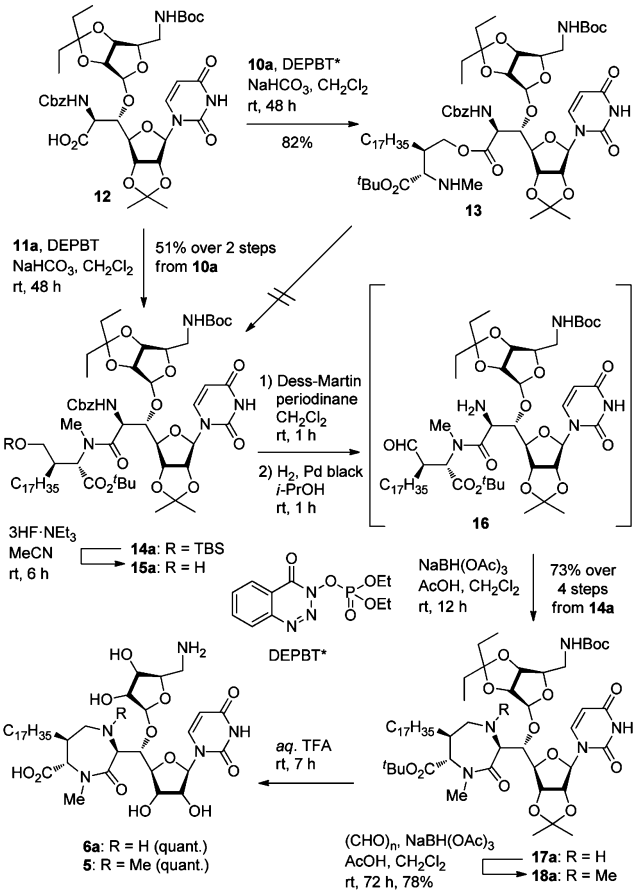
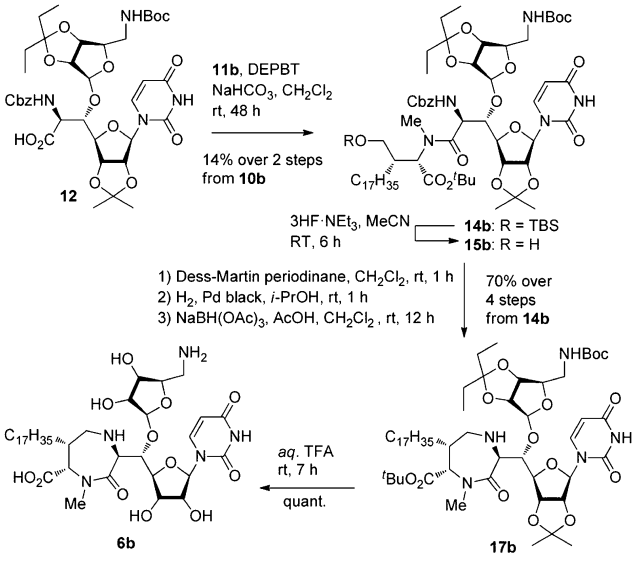
Scheme 2. Synthesis of (3‴*R*)-Carbacaprazamycins 5 and 6a



$(OAc)_3$ to provide **18a** in 78% yield. Finally, the global deprotection of **17a** and **18a** was achieved using 80% aqueous trifluoroacetic acid (TFA) to afford targets **6a** and **5**, respectively, in quantitative yields.

Diastereomer **6b** was synthesized in a manner similar to the synthesis of **6a**, as shown in Scheme 3. Note that the TBS group of **11b** was labile and easily cleaved to generate **10b**

Scheme 3. Synthesis of (3‴*S*)-Carbacaprazamycins



under the reaction conditions for coupling with **12**. The resulting hydroxyl group was then reacted with **12** to obtain a large amount of the corresponding ester. This was the reason for the low chemical yield of **14b** (14% over two steps).

With the cCPZs in hand, their inhibitory activity on the purified MraY enzyme (*S. aureus*) was then examined using a fluorescence-based MraY inhibitory assay (Table 1).[42,43] The

Table 1. Biological Evaluation of Carbacaprazamycins

	5	6a	6b	4	vancomycin
IC_{50} for MraY (nM)[a]	6.9	3.8	2.6	1.2	
MIC (μg/mL)[b]					
S. aureus ATCC 29213 (MSSA)	8	8	4	0.5	1
S. aureus SR3637 (MRSA)	8	8	4	0.5	1
E.faecalis ATCC 29212	4	8	4	0.5	1
E.faecalis SR7914 (VRE)	8	16	8	2	>64
E. facium ATCC 19434	8	8	8	1	0.5
E.faecium SR7917 (VRE)	8	16	8	1	>64

[a]The inhibitory activities of the compounds against purified MraY from *S. aureus*. [b]MICs determined by a microdilution broth method as recommended by the NCCLS. For details, see Supporting Information.

results indicated that **5**, **6a**, and **6b** were strong MraY inhibitors with IC_{50} values of 2.6−6.9 nM, although their activity was slightly lower than that of **4** (IC_{50} = 1.2 nM). The antibacterial activity of the series of compounds was then evaluated,[44] and the results are summarized in Table 1. Carbacaprazamycins **5**, **6a**, and **6b** exhibited moderate antibacterial activity against a range of Gram-positive bacterial pathogens, including *S. aureus* SR3637 (MRSA) and *E. faecium* SR7917 (VRE), with MIC values of 4−16 μg/mL. Carbacaprazamycins with the methyl group at the 6′-nitrogen atom presented slightly better antibacterial activity than did desmethyl analogues (**5** vs **6a**), as was observed in our previous SAR study of palmitoylcaprazol.[24] The stereochemistry at the 3‴ position linking to the alkyl side chain and the diazepanone has a slight impact on the antibacterial activity, and the analogues with the S configuration, which is an "unnatural" stereochemistry, at the 3‴ position were slightly more active than those with the R configuration (**6a** vs **6b**). It is suggested that one of the important factors for the biological activity of CPZs is the 3D orientation of the key three moieties, namely, the uridine, the aminoribose, and the lipophilic side chain.[31−34] Because the diazepanone is a seven-membered ring, which is inherently flexible and adopts several conformations with fewer energy differences compared with the six-membered ring, introducing the alkyl substituent on the diazepanone could easily change the conformation with less energetic cost to minimize the steric hindrance of each substituent. Presumably, this conformational adaptation of the diazepanone moiety could be one of the reasons that both diastereomers exhibited similar MraY inhibitory and antibacterial activities (see Supporting Information). The activity of carbacaprazamycins was reduced compared to that of palmitoylcaprazol (**4**) by a factor of 4−16. Presumably, replacing the ester functionality with the alkane resulted in an increase in the lipophilicity of the entire molecule. Further optimization of the lipophilic side chain would increase the antibacterial activity by modulating the lipophilicity of the molecule.

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Finally, we examined the morphology of *S. aureus* ATCC29213 (MSSA) treated with selected cCPZs **6b** using scanning electron microscopy (SEM), and Figure 3 presents

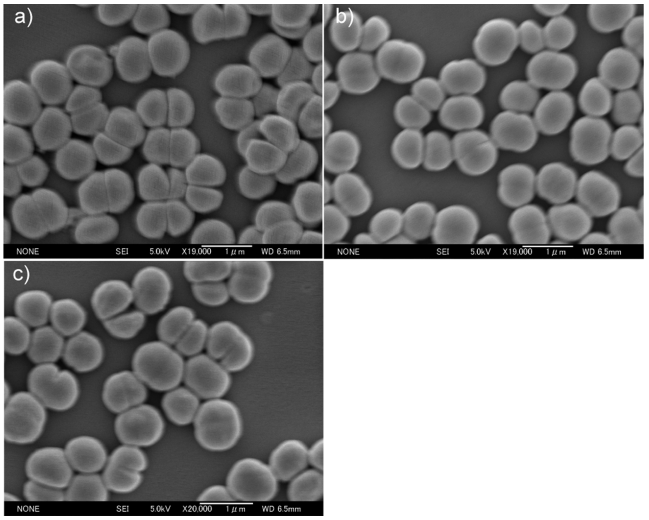

Figure 3. Scanning electron micrographs of *S. aureus* ATCC29213 treated with (a) DMSO as a control, (b) 3.2 μg/mL cCPZ (**6b**), and (c) 1.6 μg/mL vancomycin.

representative electron micrographs. Whereas the untreated cells exhibit symmetrical cell division (a), the cells treated with **6b** have a different morphological change (b). Namely, the shape of most of the cells appears to be slightly smaller around the equatorial plane. This result means that the cell division initially started on the equatorial plane but quickly halted. This change is also different from those treated with vancomycin (c). MraY catalyzes the first step of the lipid cycle in peptidoglycan biosynthesis. The observed morphological change resulting from treatment with **6b** suggested that the mode of action of caprazamycins is completely different from those of existing peptidoglycan inhibitors.

In conclusion, carbacaprazamycins **5**, **6a**, and **6b** were synthesized. Compounds **11a**,**b** were prepared in 10 steps from L-aspartic acid γ-methyl ester compared to the 19 steps required for the corresponding segment in the synthesis of **4**. This synthesis strategy also avoids the use of the base-sensitive β-acyloxy ester functionality. cCPZs retained strong MraY inhibitory activity. These analogues were also active against drug-resistant bacterial pathogens such as MRSA and VRE, although their activity was weaker than that of **4**. The effect of treatment with **6b** on morphological changes in *S. aureus* ATCC29213 was also investigated using SEM, and the results suggested that the mode of action of caprazamycins is completely different from those of existing peptidoglycan inhibitors, such as β-lactams and vancomycin. Therefore, MraY inhibitors could be a promising lead as a novel antibacterial agent effective against drug-resistant bacterial pathogens.

METHODS

Chemical Syntheses of Carbacaprazamycins. Experimental procedures are described in the Supporting Information. Analytical thin layer chromatography (TLC) was performed on Merck silica gel 60F254 plates. Normal-phase column chromatography was performed on Merck silica gel 5715. Flash column chromatography was performed on Merck silica gel 60. Dichloromethane was distilled from P_2O_5. Tetrahydrofuran, which is dehydrated stabilizer-free solvent, was purchased from Kanto Chemical Co., Inc. All reagents unless otherwise noted were directly used commercially available materials. All reactions were carried out under argon atmosphere at room temperature unless otherwise noted. Assignment was based on ^{1}H−^{1}H COSY NMR spectra. MS data were obtained on a JEOL JMS-HX101 or JEOL JMS-700TZ. The purity of all of the compounds tested for biological evaluation was confirmed to be >90% by ^{1}H NMR analysis.

Expression and Purification of Enzyme. Competent *E. coli* C43(DE3) (IMAXIO) cells were transformed with plasmid pET28b::mraY-sau or pET30a::mraY-eco. The transformants were cultured at 37 °C in 2YT supplemented with 50 μg/mL kanamycin to mid-log phase before IPTG (Sigma) was added to the culture medium at a final concentration of 1 mM. The induction was maintained at 20 °C for 16−18 h. Cells were harvested by centrifugation at 5000*g* for 10 min at 4 °C and frozen at −80 °C until they were used. After thawing, cell pellets were resuspended in 25 mM Tris-HCl at pH 7.5 containing 2 mM 2-mercaptoethanol, 150 mM NaCl, 30% glycerol, and 1 mM $MgCl_2$ (buffer A). Cells were harvested by centrifugation (5000*g* for 10 min at 4 °C) and resuspended with buffer A. Cells were broken by sonication and further treated with 10 μL of Lysonase (Novagen) per gram of cell paste. The resulting suspension was centrifuged at 200 000*g* for 30 min at 4 °C in a Beckman TL100 centrifuge. The pellet including membrane vesicles was washed three times with buffer A and then resuspended with buffer A. DDM was added at a final concentration of 17.8 mM to solubilize MraY, and the mixture was incubated at 4 °C for 2 h under mild shaking. The first supernatant (DM1) including MraY was obtained after centrifugation (200 000*g*, 30 min at 4 °C). The insoluble material was then solubilized again with buffer A containing 21.5 mM DDM. Supernatant DM2 including MraY was also recovered after centrifugation. One further round of solubilization/centrifugation was performed under the same condition (21.5 mM DDM), generating supernatant DM3. Supernatants DM1−3 were mixed and applied to 5 mL of a His-Trap HP column (GE Healthcare) that was pre-equilibrated in buffer B (25 mM Tris-HCl, pH 7.5, 300 mM NaCl, 30% glycerol, 4.0 mM DDM, 2 mM 2-mercaptoethanol). The MraY was eluted with a linear imidazole gradient from 0 to 0.5 M. The active fractions including MraY were pooled and further applied to 150 mL of a Superdex 75 prep-grade column (GE Healthcare) that was pre-equilibrated in buffer C (30 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 4 mM DDM, 2 mM 2-mercaptoethanol). The active fraction was pooled and concentrated using 10 kDa Amicon Ultra (Millipore).

Fluorescence-Based MraY Assay. Reactions were carried out in 384-well microplate. Reaction mixtures contained, in a final volume of 20 μL, 50 mM Tris-HCl (pH 7.6), 50 mM KCl, 25 mM $MgCl_2$, 0.2% Triton X-100, 8% glycerol, 100 μM C_{55}−P, and 100 μM UDP-MurNAc-dansylpentapeptide. The reaction was initiated by the addition of MraY enzyme (11 ng/5 μL/well). After 3 to 4 h of incubation at room temperature, the formation of dansylated lipid I was monitored by fluorescence enhancement (excitation at 355 nm, emission at 535 nm) by using the EnVision 2103 multilabel plate reader. The inhibitory effects of each compound were determined in the MraY assays described above. The mixtures contained 2% dimethyl sulfoxide in order to increase the solubility of the compounds.

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Antibacterial Activity Evaluation. Vancomycin-resistant *Enterococcus faecalis* SR7914 (VanA) and *Entercoccus faecium* SR7917 (VanA) and methicillin-resistant *Staphylococcus aureus* SR3637 were clinical isolates collected from hospitals in Japan and were kindly provided by Shionogi & Co., Ltd. (Osaka, Japan). MICs were determined by a microdilution broth method as recommended by the NCCLS (National Committee for Clinical Laboratory Standards, 2000, National Committee for Clinical Laboratory Standards, Wayne, PA) with cation-adjusted Mueller-Hinton broth (CA-MHB) (Becton Dickinson, Sparks, Md.). Serial 2-fold dilutions of each compound were made in the appropriate broth, and the plates were inoculated with 5×10^4 CFU of each strain in a volume of 0.1 mL. Plates were incubated at 35 °C for 20 h, and then MICs were scored.

Scanning Electron Microscope Protocol. Single colonies of *S. aureus* ATCC29213 were placed into tryptic soy broth (TSB) and shaken overnight at 30 °C. These cultures were then diluted 1/50 into 5 mL of fresh TSB and shaken at 30 °C to o.d. ≈ 0.3. DMSO (negative control), **5a** (3.2 μg/mL, DMSO solution), or vancomycin (1.6 μg/mL, DMSO solution) was added to the cultures, which continued to shake at 30 °C for 2 h. Samples were spun down (7500*g*, 8 min), and the resulting pellets were resuspended in 0.25 mL of TSB, and 0.25 mL of glutaraldehyde fixative (2% formaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4) was added to the sample. After 30 min at room temperature, the fixed samples were spun down. The pellets were washed five times with H_2O. The dried pellets were coated with Pt/Pd, and the sample images were acquired on a JEOL JSM-7400F microscope.

■ ASSOCIATED CONTENT

Supporting Information

The following file is available free of charge on the ACS Publications website at DOI: 10.1021/id5000376.

Experimental procedures for the synthesis and characterization of synthesized compounds (PDF)

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Author Contributions

M.Y. contributed to the synthesis of compounds and editing. L.S.H. contributed to the synthesis of compounds. Y.K. contributed to the establishment of the initial synthesis route. S.I. was the PI of M.Y., L.S.H., and Y.K.; made significant contributions to the writing and editing and SEM experiments; and provided significant intellectual input. A.M. was the main PI of the entire project; made significant writing and editing contributions; and provided significant intellectual input.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

cCPZ, (carbacaprazamycin); CPZ, (caprazamycin); DMAP, (*N*,*N*-dimethylaminopyridine); EDCI, (1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide hydrochloride); HOBt, (1-hydroxy-1*H*-benzotriazole); IC_{50}, (50% inhibitory concentration); MIC, (minimum inhibitory concentration); MraY, (phospho-MurNAc-pentapeptide transferase); MRSA, (*Staphylococcus aureus*); MW, (microwave); SAR, (structure−activity relationship); SEM, (scanning electron microscopy); TFA, (trifluoroacetic acid), VRE (vancomycin-resistant Enterococci)

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